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Abstract: Atypical hemolytic uremic syndrome (aHUS) is caused by several susceptibility genes. A registry including analyses of susceptibility genes, familial occurrence and genotype-phenotype correlation should provide classification insights. Registry data of 187 unrelated index patients included age at onset, gender, family history, relapse of aHUS and potentially triggering conditions. Mutation analyses were performed in the genes CFH, CD46 and CFI and in the six potential susceptibility genes, FHR1 to FHR5 and C4BP. Germline mutations were identified in 17% of the index cases; 12% in CFH, 3% in CD46 and 2% in CFI. Twenty-nine patients had heterozygous mutations and one each had a homozygous and compound heterozygous mutation. Mutations were not found in the genes FHR1-5 and C4BP. In 40% of the patients with familial HUS a mutation was found. Penetrance by age 45 was 50% among carriers of any mutation including results of relatives of mutation-positive index cases. The only risk factor for a mutation was family history of HUS ($p = 0.02$). Penetrance of aHUS in carriers of mutations is not complete. Occurrence of homo- and heterozygous mutations in the same gene suggests that the number of necessary DNA variants remains unclear. Among clinical information only familial occurrence predicts a mutation.

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Epidemiological Approach to Identifying Genetic Predispositions for Atypical Hemolytic Uremic Syndrome

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Summary

Atypical hemolytic uremic syndrome (aHUS) is caused by several susceptibility genes. A registry including analyses of susceptibility genes, familial occurrence and genotype-phenotype correlation should provide classification insights.

Registry data of 187 unrelated index patients included age at onset, gender, family history, relapse of aHUS and potentially triggering conditions. Mutation analyses were performed in the genes *CFH*, *CD46* and *CFI* and in the six potential susceptibility genes, *FHR1 to FHR5* and *C4BP*.

Germline mutations were identified in 17% of the index cases; 12% in *CFH*, 3% in *CD46* and 2% in *CFI*. Twenty-nine patients had heterozygous mutations and one each had a homozygous and compound heterozygous mutation. Mutations were not found in the genes *FHR1-5* and *C4BP*. In 40% of the patients with familial HUS a mutation was found. Penetrance by age 45 was 50% among carriers of any mutation including results of relatives of mutation-positive index cases. The only risk factor for a mutation was family history of HUS ($p = 0.02$).

Penetrance of aHUS in carriers of mutations is not complete. Occurrence of homo- and heterozygous mutations in the same gene suggests that the number of necessary DNA variants remains unclear. Among clinical information only familial occurrence predicts a mutation.

Keywords: Complement factor H (CFH), CD46, complement factor I (CFI), hemolytic uremic syndrome

Introduction

Hemolytic uremic syndrome (HUS) is characterised by non-immunogenic hemolytic anemia, low platelet count and deterioration of renal function. Laboratory findings include decreased hemoglobin, erythrocytes, platelets, haptoglobin, fragmentocytes in the blood smear, and negative Coombs test. Thrombotic microangiopathy is the histomorphologic correlate. Atypical hemolytic uremic syndrome (aHUS), the

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counterpart of classic HUS represents most cases of HUS in adulthood. In contrast to typical HUS which is induced by infections of bacteria producing Shiga like toxins, mostly by enterohaemorrhagic escherichia coli strain O157:H7, aHUS is not associated with diarrhoea (Kaplan et al., 1998; George, 2006). The clinical classification of aHUS is based on associated conditions, mainly with exposure to certain drugs like ovulation inhibitors or platelet aggregation inhibitors, with various disorders like acute infections of the respiratory and gastrointestinal tracts and changes of life cycle like pregnancy (George, 2006). In contrast to classic HUS, plasma exchange and substitution by fresh frozen plasma is an established therapy for aHUS and has considerably ameliorated the poor spontaneous prognosis of aHUS (Rock et al., 1991). However, patients with aHUS and endstage renal failure have a further burden of recurrence after kidney transplantation (25-60%) (Agarwal et al., 1995; Artz et al., 2003) and transplant failure after recurrence of HUS (90%) (Conlon et al., 1996; Miller et al., 1997; Lahlou et al., 2000; Kavanagh et al., 2008).

New insights into the molecular genetics and the pathophysiological background of aHUS have been provided in recent years. Genetic linkage analyses of families with HUS gave evidence for a susceptibility gene on chromosome 1q32 which was subsequently identified as the gene encoding factor H (*CFH*) (Warwicker et al., 1998). Subsequently, another component of the complement cascade, the *CD46* gene encoding the membrane cofactor protein (MCP) has also been identified as a susceptibility gene for aHUS (Noris et al., 2003; Richards et al., 2003). Finally, the family of susceptibility genes for HUS has been extended to the gene encoding the complement factor I, *CFI* (Fremeaux-Bacchi et al., 2004; Kavanagh et al., 2005; Caprioli et al., 2006). Interestingly at the same chromosomal locus 1q32 a clustering of genes encoding proteins involved in the complement system has been found. These genes form the RCA (regulators of complement activation) cluster and comprise among others the genes *FHR-1*, *FHR-2*, *FHR-3*, *FHR-4*, *FHR-5, CFH, CD46* and *C4BP* (Rey-Campos et al., 1988).

For clinicians, primarily nephrologists, the frequency of carriers of germline mutations in susceptibility genes among patients with aHUS and potential correlations with clinical features are of great interest. We have addressed these questions in regard to the aHUS patients of our registry for the genes *CFH*, *CD46* and *CFI*. Furthermore, in order to identify other susceptibility genes, we screened registrants for germline mutations in the genes *FHR 1-5* and *C4BP alpha* and *beta*.

Three susceptibility genes which were recently described as being associated with HUS, *C3*, factor B *(FB)* and thrombomodulin, were not considered for molecular analysis in this study (Goicoechea de Jorge et al., 2007; Fremeaux-Bacchi et al., 2008; Delvaeye et al., 2009).

Materials and Methods

The Registry of Patients with aHUS

The registry for aHUS based in Freiburg, Germany was established in 1998 for German speaking countries including Germany, Switzerland, Austria and northeast Italy (Alto Adige) (Neumann et al., 2003). Initially dedicated to adults, the registry was later extended to patients in childhood and adolescence. Index cases with aHUS who provided EDTA anticoagulated blood, demographic and clinical data were included. Among subjects with a positive family history for HUS, the affected relatives were asked for the same items. Patient data included haemoglobin, platelet count, serum creatinine, lactate dehydrogenase and haptoglobin as well as fragmentocytes in the blood smear and negative Coombs test in the acute episode. Clinical events preceding the acute HUS episode were recorded; these included acute infections, cancer, autoimmune diseases, intake of drugs such as platelet aggregation inhibitors, ovulation inhibitors, chemotherapeutics or immunosuppressive medication or special diet components like quinine, and life cycle changes such as pregnancy or postpartum period. In addition we recorded renal biopsy findings in the acute phase of HUS and relapse episodes after the acute phase. We re-evaluated the registrants for a previous infection by the major shiga-like toxin-producing bacteria E. coli strain O157:H7 and measured serum C3 and factor H. We excluded patients with positive E. coli serology, patients without impairment of renal function, patients with neurological deficits which were regarded as signs for TTP and patients who developed HUS only after kidney transplantation.

Special attention was put on patients thought to be at high risk of developing aHUS. This group consisted of patients with familial HUS, those with relapsing HUS after remission documented by renal function, platelet count and haptoglobin after treatment by plasma membrane separation and substitution of fresh frozen plasma, and patients with aHUS in young age defined as 40 years or younger. Finally, we contacted all patients initially and during follow up for outcome and recorded, in particular, occurrence of endstage renal failure.

Mutation Screening in Susceptibility Genes and Candidate Genes

All patients were tested for susceptibility genes *CFH*, *CFI* and *CD46* (Kavanagh et al., 2008). For *CFH* analyses until 2004 we used single strand confirmation polymorphism (SSCP) and after this time denaturing high performance liquid chromatography was used (DHPLC, WAVE system, model 3500 HT, Transgenomic, Glasgow, United Kingdom). The genes *CD46* and *CFI* were exclusively analyzed by DHPLC. In addition we analyzed the potential candidate genes *FHR-1*, *FHR-2*, *FHR-3*, *FHR-4*, *FHR-5* and *C4BP* by DHPLC. Direct sequencing was performed if DHPLC showed abnormal chromatographies or SSCP aberrant bands.

Quality Check for Sensitivity and Specificity of SSCP and DHPLC

Direct sequencing is the gold standard for intraexonic mutation analysis whereas generic sensitivity of 86-100% with SSCP and 93-100% with DHPLC are inferior (Xiao & Oefner, 2001; Bettinaglio et al., 2002), but more cost effective. Regarding SSCP, we chose 40 index cases in which SSCP showed normal results for *CFH*. Regarding DHPLC, we chose 13 index cases in which DHPLC showed normal results for *CFH* and 40 index cases in which DHPLC showed normal results for *CD46* and *CFI*. Direct sequencing was performed to confirm results of both methods in 40 (SSCP) and 13 (DHPLC) index cases for the gene *CFH*, and in 40 index cases (DHPLC) for the genes *CD46* and *CFI*. Results showed normal findings in all genes. Furthermore, we compared sensitivity of SSCP versus DHPLC exclusively in those *CFH* exons in which SSCP showed a DNA variant. Again, DHPLC was abnormal in all these SSCP samples. In summary, sensitivity of SSCP and DHPLC in *CFH* analyses is identical and 100% in this quality check. Therefore, we did not re-investigate other index cases which were analyzed by SSCP.

Interpretation of DNA Variants

DNA variants creating stop codons or truncation of the putative protein due to splice site alteration as well as intra-exonic deletions or insertions were regarded as pathogenetically relevant, i.e. mutations. In contrast, this is not self-evident for missense variants which were, therefore, tested in 100 healthy blood-donor controls (200 chromosomes). Missense variants were regarded as mutations, if they were not observed in the controls. For each missense mutation we checked the affected codons for evolutionary conservation among species using the EPO Multiple Alignment Analysis provided by ENSEMBL Browser (Flicek et al., 2008). Furthermore, we conducted *in silico* analysis based on sequence similarity analyses, the physical properties of amino acids, and the structure and function of human proteins using the SIFT predictor Software (Ng & Henikoff, 2006). For compound heterozygous and homozygous missense mutations an additional 1000 controls were screened.

Genetic Testing of Family Members

Relatives of patients with identified mutations were offered mutation screening. Registered clinical data of relatives included the same items as for the index cases.

Statistical Analysis

We tested the hypothesis of an association between the clinical/demographic data and presence of any mutation in patients in which all three genes (*CFH*, *CD46* and *CFI*) were tested. Further, we assessed the association between the clinical/demographic data and the presence of a mutation for all the patients studied for each gene separately. These analyses were done using a two-sided Fisher's exact test. Age-dependent penetrance was estimated using the Kaplan-Meier method.

This study was approved by the Ethical Committee of the Freiburg University Medical Center. All patients gave written informed consent.

Results

The analysis included 187 unrelated index cases with aHUS, 112 females and 75 males, age 3 months to 78 years (mean 30 years) at diagnosis ($\pm$0.08) of the disease. Of the 187 cases, 31 probands were mutation positive. These 31 index patients carried a mutation in one of the genes, *CFH*, *CD46* or *CFI*. Twenty-two index cases (12%) showed *CFH* mutations, 6 index cases (3%) showed *CD46* mutations, and 3 index cases (2%) cases were carriers of a *CFI* mutation.

Further, we included in the analyses 48 relatives from 24 of the 31 mutation positive index cases, demonstrating the mutations in 23 of these 48. In five of these 24 index cases familial HUS was present. Mutations could be demonstrated in eight relatives of four families (Fig. 1, panel A).

DNA Variants of the *CFH* Gene

There were 33 total *CFH* DNA variants, which included 22 different *CFH* mutations and 11 polymorphisms (Tables 1 and 2). The mutations comprised 16 missense, one splice site and two stop codon mutations, and two small deletions of three and four nucleotides respectively. All mutations were located in exons 14-23 of the *CFH* gene. Three of the mutations have not yet been reported. The already reported *CFH* c.3701 C>T mutation was found in one case together with the *CFH* c.3135 A>T mutation. In another case, the *CFH* c.3701 C>T mutation was the only mutation. The *CFH* c.2770 T>A (*P*.Y899X) mutation was only found in one case from consanguineous parents and was homozygous in the index case. Twenty-two of 187 patients (12%) had *CFH* mutations.

DNA Variants of the *CD46* Gene

A total of seven different intra-exonic and splice site DNA variants have been found in the *CD46* gene. These comprised five mutations and two polymorphisms (Tables 1 and 2). The mutations were one stop codon, one splice site and two missense mutations, and one small deletion of one nucleotide. One of the mutations has not yet been reported. Six of 187 patients (3%) had *CD46* mutations; one mutation was seen in two index cases.

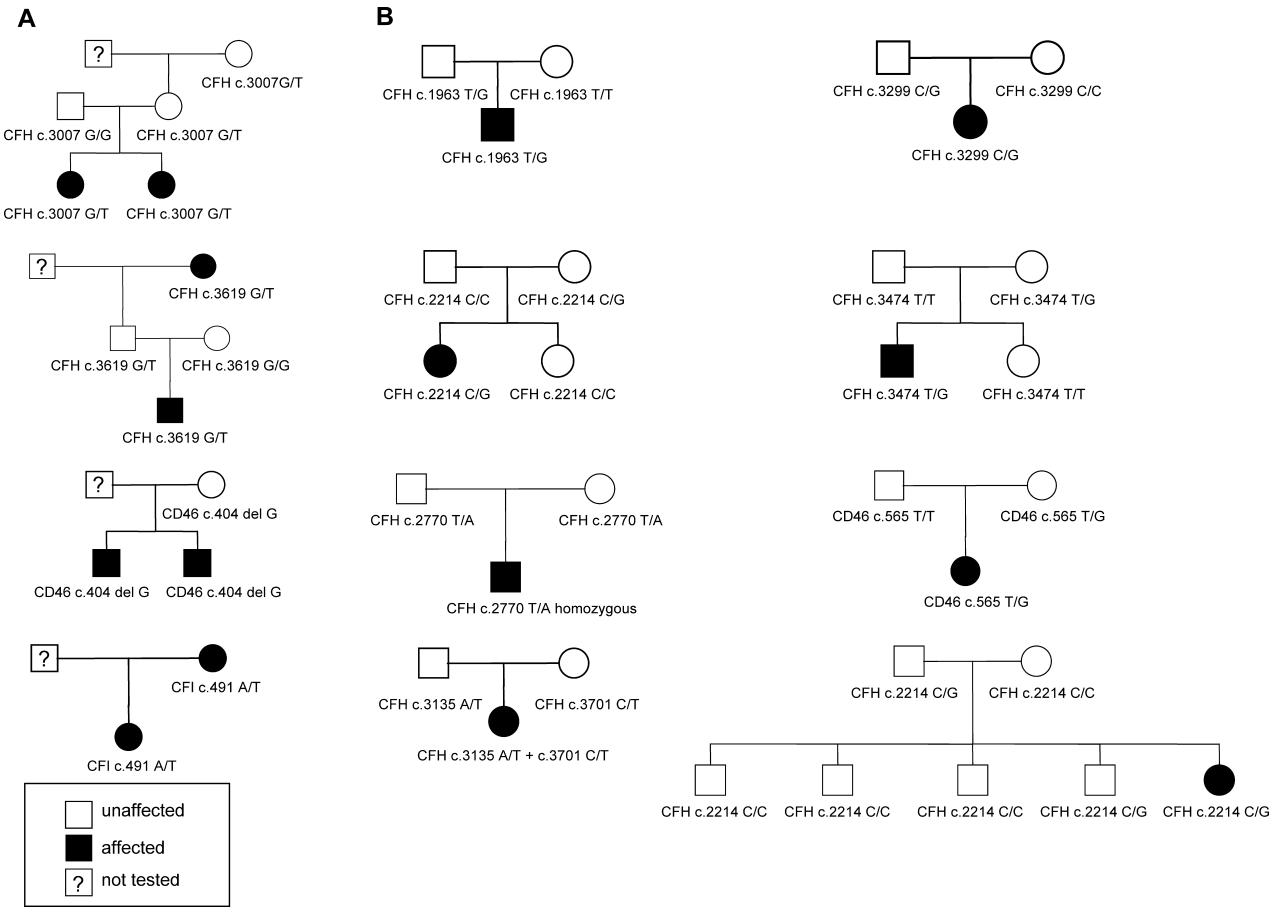

Figure 1 Pedigrees of mutation-positive index cases with familial HUS (A: four families) and without familial HUS (B: eight families).

DNA Variants of the *CFI* Gene

A total of five different intra-exonic and splice site variants were found in the *CFI* gene, three mutations and two polymorphisms (Tables 1 and 2). The mutations comprised three missense mutations. Two mutations have not yet been reported. Three of 187 (2%) cases were carriers of a *CFI* mutation.

DNA Variants of the Genes *FHR 1-5* and *C4BP*

No pathogenic relevant variants were found in these six genes. Identified polymorphisms are listed in Table 2.

Familial HUS

A total of 15 index cases had a family history of HUS; mutations were detected in six (40%) of these. In five mutation positive cases blood DNA was available from 11 relatives including seven parents. In these relatives, eight additional mutation carriers were found (Fig. 1, panel A).

Relatives of Patients with Mutations

In 25 index cases with a mutation, there was no family history of HUS. From 19/25 of these mutation carriers a total of 37 relatives were available for genetic testing. Both parents were available for genetic testing from 11 index cases. For six cases the mutation was found in one parent and for two cases a mutation was found in both parents (Fig. 1, panel B), whereas in three cases *de novo* mutations occurred, since none of the parents were carriers; haplotype analysis confirmed paternity.

Re-interviews and investigations showed normal serum creatinine levels and no evidence for HUS in these ten newly recognized carriers. For the index case who showed a compound heterozygous mutation, *CFH* c.3701 C>T and c.3135 A>T, the first mutation was found in the mother, the second in the father, neither of whom ever had HUS. For the index case with the homozygous mutation, *CFH*

Table 1 New and previously published mutations in the genes CFH, CD46 and CFI.

SCR	Exon	Nucleotide	AA	Mutationtype	Previously described
CFH gene mutations					
11	14	1963 T>G	C630W	Missense	(Neumann et al., 2003)
12	15	2214 C>G	S714X	Nonsense	(Neumann et al., 2003)
14	17	2576 G>T	V835L	Missense	(Saunders et al., 2006)
14	17	2621 G>A	E850K	Missense	(Neumann et al., 2003)
15	18	2770 T>A homoz.	Y899X	Nonsense	(Caprioli et al., 2006)
16	19	3007 G>T	W978C	Missense	(Neumann et al., 2003)
17	20	3135 A>T	Y1021F	Missense	(Neumann et al., 2003)
17	20	3200 T>C	C1043R	Missense	(Neumann et al., 2003)
18	21	3299 C>G	Q1076E	Missense	(Richards et al., 2001; Neumann et al., 2003)
19	22	3474 T>G	V1134G	Missense	(Neumann et al., 2003)
19	22	3478 G>C	E1135D	Missense	New
19	22	3497 T>G	Y1142D	Missense	(Neumann et al., 2003)
19	22	3542 T>C	W1157R	Missense	(Neumann et al., 2003)
19	22	3566 + 1 G>A		Splice-Defect	(Neumann et al., 2003)
20	23	3619 G>T	R1182S	Missense	New
20	23	3620 T>C	W1183R	Missense	New
20	23	3645 C>T	S1191L	Missense	(Richards et al., 2001; Heinen et al., 2006)
20	23	3701 C>T	R1210C	Missense	(Caprioli et al., 2001; Sanchez-Corral et al., 2002)
20	23	3719 Δ del ACA	In-Frame Deletion	Frameshift	(Neumann et al., 2003)
20	23	3749 C>T	P1226S	Missense	(Neumann et al., 2003)
20	23	3767 del AGAA	X1232FfsX38	Frameshift	(Neumann et al., 2003)
CD46 gene mutations					
1	1	104 G>A	C35Y	Missense	(Caprioli et al., 2006)
1	1	175 C>T	R59X	Nonsense	(Caprioli et al., 2006)
1	1	286 + 2 T>G		Splice-Defect	(Fremeaux-Bacchi et al., 2006)
2	3	404 del G	G135VfsX13	Frameshift	New
3	4	565 T>G	Y189D	Missense	(Fremeaux-Bacchi et al., 2006)
CFI gene mutations					
	4	485 G>A	G162D	Missense	New
	4	491 A>T	D164V	Missense	New
	5	772 G>A	A258T	Missense	(Vyse et al., 1996)

c.2770 T>A, the mutation was present in both parents in heterozygous form. From two index cases a sibling was available for testing who were both positive, but without a history of HUS.

Penetrance of HUS in *CFH*, *CD46*, and *CFI* Germline Mutation Carriers

Age-dependent HUS penetrance was estimated for all 54 subjects carrying a *CFH, CD46,* or *CFI* mutation (Fig. 2 Panel A). These data demonstrate an incomplete penetrance of HUS in mutation carriers with a manifestation of HUS in 50% of the carriers by age 45. The data are more robust for *CFH,* since 39 patients had mutations in this gene compared to ten and five with mutations of the genes *CD46,* and *CFI* respectively, but for all genes penetrance reached 50% in the 5th decade of life (Fig. 2 Panel B, C and D).

Genotype-Phenotype Correlations – Predictors For Mutations in any Susceptibility Gene

Regarding conditions which predispose or potentially predispose to aHUS (Table 3), a germline mutation was identified in six of 15 index cases with familial HUS ($p = 0.02$). Considering the distribution of mutation positive cases through different age intervals (Fig. 3), the proportion of mutation positive cases was higher in patients at age $\leq$40 (28/143; 20%) in contrast to those who were older (4/44; 9%), a trend toward statistical significance ($p = 0.06$). Relapsing HUS occurred in 53 index cases of whom ten (19%) had a mutation ($p = 0.66$).

Regarding conditions which potentially triggered the manifestation of aHUS, 91 index cases had either concomitant acute or chronic inflammatory diseases (43 cases), malignancies (seven cases), pharmacological treatment with ovulatory inhibitors (11 cases) or other drugs (12 cases), pregnancy or

Table 2 *CFH*, *CD46*, *CFI*, *FHR-1*, *FHR-5* and *C4BP* gene polymorphisms in patients of the Freiburg registry.

SCR	Exon	Nucleotide	AA	Occurrence in Healthy probands in %
CFH gene polymorphisms				
	1	80 C>G	Intron	
1	2	257 G>A	V62I	2*/3**
5	7	994 C>A	A307A	
7	9	1277 C>T	H402Y	49*/36**
8	11	1492 G>A	A473A	
11	14	2089 A>G	Q672Q	
15	18	2707 C>T	H878H	2*/0**
16	19	2881 G>T	E936D	18*/2**
16	19	2923 G>T	Q950H	2*/0**
16	19	2881 + 2923	E936D+Q950H	2*/0**
18	21	3211 C>T	T1046T	
18	21	3221 A>T	N1050Y	6*/0**
CD46 gene polymorphisms				
2	3	417 A>G	L139L	2*/0**
4	5	718 T>C	S240P	3*/1**
CFI gene polymorphisms				
	6	782 G/A	G261D	1*/0**
	6	804 G>A	G268G	3*/0**
FHR-1 gene polymorphisms				
4	4	578 C>T	H157Y	28*/29**
4	4	584 C>G	L159V	28*/29**
4	4	632 G>C	E175Q	28*/29**
4	4	697 A>G	T196T	28*/29**
5	5	778 G>A	P223P	14*/11**
FHR-5 gene polymorphisms				
7	7	1067 G>A	R356	25*/0**
C4BP-alpha gene polymorphisms				
	2	11 C>A	P4Q	10*/0**
5	8	899 T>C	I300T	51*/56**
C4BP-beta gene polymorphisms				
3	4	462 C>T	N154N	47*/0**

*heterozygote/**homozygote.

postpartum period (27 cases) prior to onset of HUS. Among these 91 index cases, 16 (18%) had a mutation. The frequency of mutations with concomitant diseases was 21% (9/43). One mutation was found in one patient with malignancy. Significant association was not found between the presence of mutations and any of the specific triggering factors of HUS (Table 3). No significant association was observed between these conditions and germline mutations in the *CFH*, *CD46* and *CFI* genes (Table 4).

Renal outcome information was available for 183/187 registrants including 30/31 mutation carriers. Endstage renal disease (ESRD) developed in 91 of 153 registrants without a mutation and in 20 of 30 registrants with a mutation in *CFH*, *CD46* or *CFI* ($p = 0.54$). Regarding the different genes, ESRD was observed in carriers of a *CFH* ($n = 15$), *CD46* ($n = 2$) and *CFI* ($n = 3$) mutation (Tables 3 and 4).

Discussion

We present a study on 187 unrelated index patients with aHUS screened for germline mutations in the genes *CFH*, *CD46* and *CFI*. A total of 31 index cases harbour a germline mutation, 22 in the *CFH* gene, six in the *CD46* gene and three in the *CFI* gene. About one fifth (19%) of detected mutations in this registry have not yet been described (Saunders et al., 2006). The mutation detection rate is 17% and thus somewhat lower compared to the reports of 22% – 51% from the groups in Newcastle, Paris, Madrid and Bergamo (Esparza-Gordillo et al., 2005; Fremeaux-Bacchi et al., 2005; Caprioli et al., 2006; Fremeaux-Bacchi et al., 2006). The detection rate in these groups might be biased through the pre-selection of cases considered as "high risk for inheritance". These are cases with positive family history, relapsing HUS and young age at diagnosis. Our results are based on unselected registrants and are likely to represent the true detection rate in patients with aHUS. Our data confirmed that mutations in the genes *CD46* and *CFI* are less frequent than those in the *CFH* gene.

Recently, three additional susceptibility genes for aHUS have been described (Goicoechea de Jorge et al., 2007; Fremeaux-Bacchi et al., 2008; Delvaeye et al., 2009). The genes factor B *(FB)* and *C3* have been shown to be mutated in two and 11 index cases respectively so far. These genes were exclusively mutated in patients with aHUS and decreased serum C3 levels. The third gene is the gene thrombomodulin (Delvaeye et al., 2009). We did not consider performing mutation screening in these newly identified genes since we focused our study on the genes *CFH,* CD46 and *CFI* and also because of the high costs for a large cohort such as ours.

Two cases in this series are particularly remarkable. One patient showed the mutation *CFH* c.2770 T>A. This is a stop codon mutation which predicts a truncated protein and is seemingly doubtless pathogenic. In our index case, however, this mutation is homozygous and inherited from both parents who are heterozygous and without manifestation of HUS. The second case shows two *CFH* mutations, c.3135 A>T and c.3701 C>T, both of the missense type. According to the results in both parents, who each were carriers of one mutation respectively, this index case is compound heterozygous. Of note, one of the two mutations, c.3701 C>T, was the only mutation in another index case. In fact, similar cases are reported in the literature (Caprioli et al., 2006; Fremeaux-Bacchi et al., 2006). It remains unclear how to understand

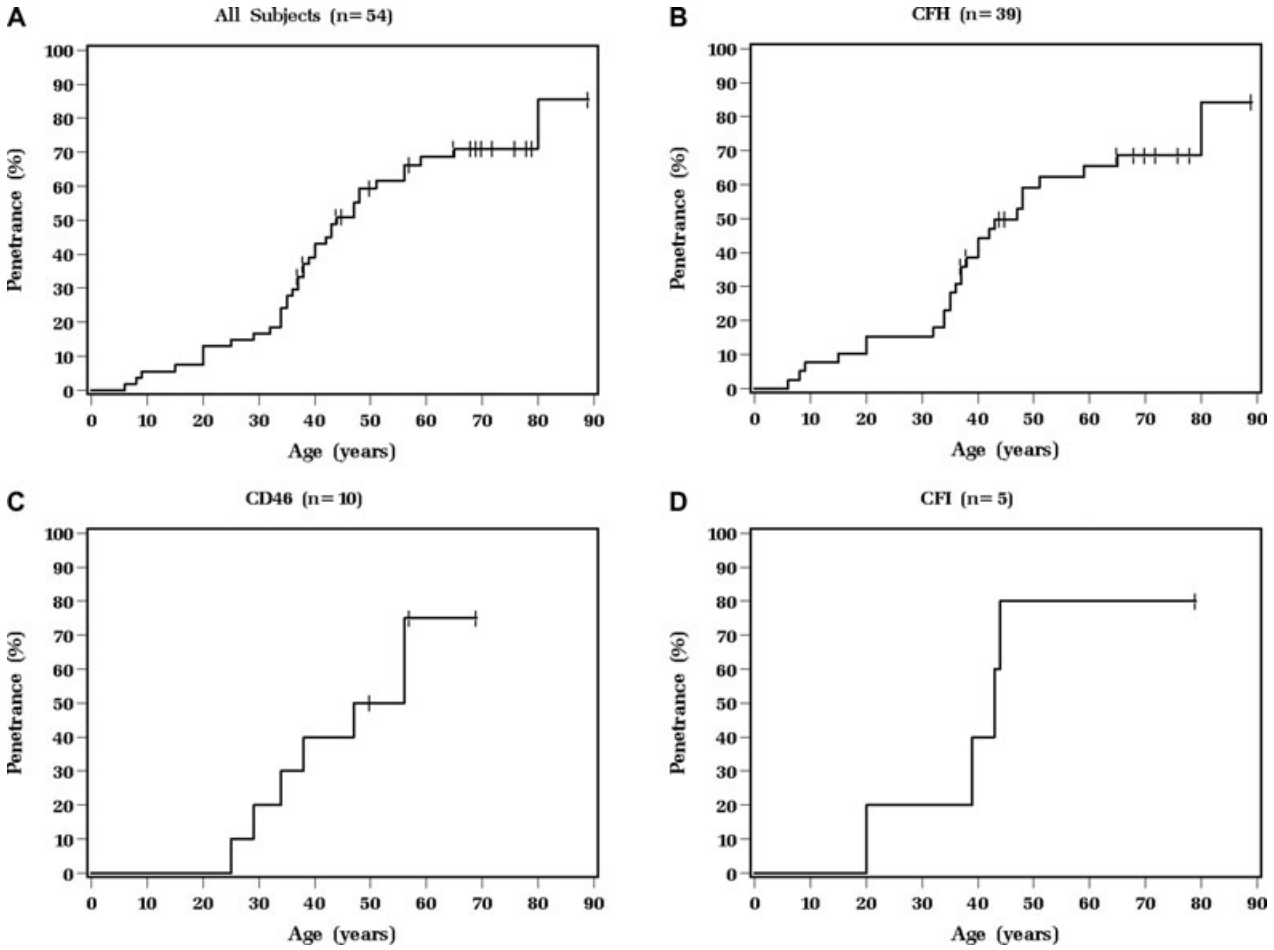

Figure 2 Penetrance of HUS in germline mutation carriers of the genes *CFH*, *CD46*, and *CFI*.

Table 3 Clinical phenotype and presence/absence of any mutation.

Patients characteristics	Mutation neg (156)	Mutation pos (31)	total	p
Predisposing conditions				
Age ≤40	115	28	143	0.06
Relapsing HUS	43	10	53	0.66
Family History for HUS	9	6	15	0.02
Potentially triggering conditions				
Ovulation Inhibitors	7	4	11	0.09
Other Drugs	11	1	12	0.69
Pregnancy/Post Partum	24	3	27	0.58
Other Disorders	40	10	50	0.51
Outcome				
ESRD	91	20	111	0.54

these findings which considerably weaken the concept that a single germline mutation defines a disease predisposition and encompasses this powerful condition. The dosage of required predisposing events thus became unclear. The current understanding of pathogenesis of aHUS considers the mutations themselves as one of the susceptibility factors but not the entire cause of the disease. In the pathogenesis endothelial insults due to infections, drugs, pregnancy or other events are required for initiation of uncontrolled complement activation leading to further damage to blood vessels and thrombotic microangiopathy (Fang et al., 2008).

Notably, the variant *CD46* c.718 T>C (corresponding to RefSeq NM_172359.1), which was previously described as a mutation by Richards et al. (2003) was also found in one of our patients and in four controls. Three of the controls were heterozygous and one was homozygous for this variant. We then tested to determine whether the nucleotide is highly conserved among different species, and whether the resulting amino acid change is predicted to be deleterious, in order to clarify the pathogenic role of these variants. The nucleotide was conserved among species and the amino acid change predicted to be tolerant. Taking the calculated result together with the 4% incidence in our control samples, we classified this variant as a polymorphism.

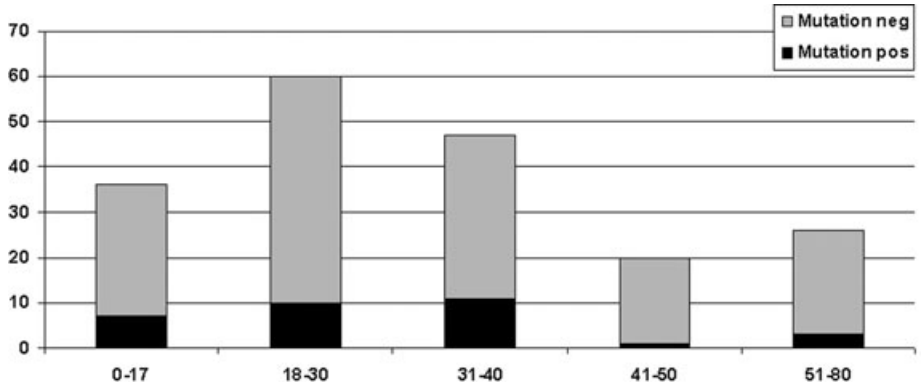

Figure 3 Distribution of mutation-positive cases through different age intervals.

Table 4 Clinical phenotype and presence/absence of specific gene mutations.

	CFH gene mutations			*CD46* gene mutations			*CFI* gene mutations		
	neg (165)	pos (22)	p	neg (181)	pos (6)	p	neg (184)	pos (3)	p
Patients characteristics									
Age ≤40	123	20	0.11	137	6	0.34	141	2	0.56
Relapsing HUS	48	5	0.62	49	4	0.06	52	1	>0.99
Family History for HUS	12	3	0.39	13	2	0.08	14	1	0.22
Potentially triggering conditions									
Ovulation Inhibitors	8	3	0.13	10	1	0.31	11	0	>0.99
Other Drugs	12	0	0.37	12	0	>0.99	11	1	0.18
Pregnancy/Post Partum	25	2	0.75	27	0	0.60	26	1	0.38
Other Disorders	43	7	0.61	48	2	0.66	49	1	>0.99
Outcome									
ESRD	96	15	0.35	109	2	0.21	108	3	0.28

Based on a total of 54 mutation carriers we estimated the penetrance for HUS. Remarkably, none of the newly recognized carriers had any evidence for an episode of HUS and kidney function was normal. Consequently, the penetrance was far from complete and reached only 50% by the age of 40-50 years for all carriers of any mutation in any of the three genes as well as for carriers of one of the three genes separately (Fig. 2 Panels A-D).

With an increase in the number of susceptibility genes involved in the genetic predisposition for aHUS, it becomes important to determine which subjects are at higher risk of having a germline mutation and which gene should be analyzed. We focused this issue by using three potential mutation predictors: familial HUS, relapsing HUS and young age at onset (age of ≤40 years). Family history was the only risk factor for the presence of an inherited disorder ($p = 0.02$). Interestingly, 25 of the 31 (81%) mutation carriers did not present with positive family history. Furthermore, it is of interest that in nine of 15 cases (60%) with positive family history the responsible gene was not identified in this study, indicating that the inheritance pattern for aHUS is still not completely clarified. Relapsing aHUS, seen in 54 of our index cases was not found to be associated with presence of an inherited disorder ($P = 0.66$). However, considering the three genes separately, an association toward significance could be observed for relapsing HUS and *CD46* germline mutations ($p = 0.06$). The age of ≤40 years is a cut-off of potential interest ($p = 0.06$) and needs to be confirmed by other studies.

Finally renal outcome with endstage renal disease was observed in patients without mutations as well as in patients with mutations in the susceptibility genes *CFH*, *CD46* and *CFI* and thus, endstage renal disease does not represent a predictor for such mutations.

In summary, this large registry with 187 index cases of patients demonstrates germline mutations within one of three susceptibility genes in 17% of patients with atypical HUS. Twenty-three of 54 relatives were also mutation carriers but without evidence for aHUS episodes, which lowers the penetrance for the disease to 50% in the 5th decade of life. The only risk factor for a germline mutation is family history for

aHUS, whereas diagnosis at age ≤40 years or relapsing HUS do not reach statistical significance.

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References

Agarwal, A., Mauer, S. M., Matas, A. J. & Nath, K. A. (1995) Recurrent hemolytic uremic syndrome in an adult renal allograft recipient: current concepts and management. *J Am Soc Nephrol* **6**, 1160–1169.

Artz, M. A., Steenbergen, E. J., Hoitsma, A. J., Monnens, L. A. & Wetzels, J. F. (2003) Renal transplantation in patients with hemolytic uremic syndrome: high rate of recurrence and increased incidence of acute rejections. *Transplantation* **76**, 821–826.

Bettinaglio, P., Galbusera, A., Caprioli, J., Orisio, S., Perna, A., Arnoldi, F., Bucchioni, S. & Noris, M. (2002) Single Strand Conformation Polymorphism (SSCP) as a quick and reliable method to genotype M235T polymorphism of angiotensinogen gene. *Clin Biochem* **35**, 363–368.

Caprioli, J., Bettinaglio, P., Zipfel, P. F., Amadei, B., Daina, E., Gamba, S., Skerka, C., Marziliano, N., Remuzzi, G. & Noris, M. (2001) The molecular basis of familial hemolytic uremic syndrome: mutation analysis of factor H gene reveals a hot spot in short consensus repeat 20. *J Am Soc Nephrol* **12**, 297–307.

Caprioli, J., Noris, M., Brioschi, S., Pianetti, G., Castelletti, F., Bettinaglio, P., Mele, C., Bresin, E., Cassis, L., Gamba, S., Porrati, F., Bucchioni, S., Monteferrante, G., Fang, C. J., Liszewski, M. K., Kavanagh, D., Atkinson, J. P. & Remuzzi, G. (2006) Genetics of HUS: the impact of MCP, CFH, and IF mutations on clinical presentation, response to treatment, and outcome. *Blood* **108**, 1267–1279.

Conlon, P. J., Brennan, D. C., Pfaf, W. W., Finn, W. F., Gehr, T., Bollinger, R. R. & Smith, S. R. (1996) Renal transplantation in adults with thrombotic thrombocytopenic purpura/haemolytic-uraemic syndrome. *Nephrol Dial Transplant* **11**, 1810–1814.

Delvaeye, M., Noris, M., De Vriese, A., Esmon, C. T., Esmon, N. L., Ferrell, G., Del-Favero, J., Plaisance, S., Claes, B., Lambrechts, D., Zoja, C., Remuzzi, G. & Conway, E. M. (2009) Thrombomodulin mutations in atypical hemolytic-uremic syndrome. *N Engl J Med* **361**, 345–357.

Esparza-Gordillo, J., Goicoechea De Jorge, E., Buil, A., Carreras Berges, L., Lopez-Trascasa, M., Sanchez-Corral, P. & Rodriguez De Cordoba, S. (2005) Predisposition to atypical hemolytic uremic syndrome involves the concurrence of different susceptibility alleles in the regulators of complement activation gene cluster in 1q32. *Hum Mol Genet* **14**, 703–712.

Fang, C. J., Richards, A., Liszewski, M. K., Kavanagh, D. & Atkinson, J. P. (2008) Advances in understanding of pathogenesis of aHUS and HELLP. *Br J Haematol* **143**, 336–348.

Flicek, P., Aken, B.L., Beal, K., Ballester, B., Caccamo, M., Chen, Y., Clarke, L., Coates, G., Cunningham, F., Cutts, T., Down, T., Dyer, S. C., Eyre, T., Fitzgerald, S., Fernandez-Banet, J., Gräf, S., Haider, S., Hammond, M., Holland, R., Howe, K.L., Howe, K., Johnson, N., Jenkinson, A., Kähäri, A., Keefe, D., Kokocinski, F., Kulesha, E., Lawson, D., Longden, I., Megy, K., Meidl, P., Overduin, B., Parker, A., Pritchard, B., Prlic, A., Rice, S., Rios, D., Schuster, M., Sealy, I., Slater, G., Smedley, D., Spudich, G., Trevanion, S., Vilella, A. J., Vogel, J., White, S., Wood, M., Birney, E., Cox, T., Curwen, V., Durbin, R., Fernandez-Suarez, X. M., Herrero, J., Hubbard, T. J., Kasprzyk, A., Proctor, G., Smith, J., Ureta-Vidal, A. & Searle, S. (2008) ENSEMBL 2008. *Nucleic Acids Res* **36**, D707–714.

Fremeaux-Bacchi, V., Dragon-Durey, M. A., Blouin, J., Vigneau, C., Kuypers, D., Boudailliez, B., Loirat, C., Rondeau, E. & Fridman, W. H. (2004) Complement factor I: a susceptibility gene for atypical haemolytic uraemic syndrome. *J Med Genet* **41**, e84.

Fremeaux-Bacchi, V., Kemp, E. J., Goodship, J. A., Dragon-Durey, M. A., Strain, L., Loirat, C., Deng, H. W. & Goodship, T. H. (2005) The development of atypical haemolytic-uraemic syndrome is influenced by susceptibility factors in factor H and

membrane cofactor protein: evidence from two independent cohorts. *J Med Genet* **42**, 852–856.

Fremeaux-Bacchi, V., Miller, E. C., Liszewski, M. K., Strain L., Blouin, J., Brown, A. L., Moghal, N., Kaplan, B. S., Weiss, R. A., Lhotta, K., Kapur, G., Mattoo, T., Nivet, H., Wong, W., Gie, S., Hurault De Ligny, B., Fischbach, M., Gupta, R., Hauhart, R., Meunier, V., Loirat, C., Dragon-Durey, M. A., Fridman, W. H., Janssen, B. J., Goodship, T. H. & Atkinson, J. P. (2008) Mutations in complement C3 predispose to development of atypical hemolytic uremic syndrome. *Blood* **112**, 4948–4952.

Fremeaux-Bacchi, V., Moulton, E. A., Kavanagh, D., Dragon-Durey, M. A., Blouin, J., Caudy, A., Arzouk, N., Cleper, R., Francois, M., Guest, G., Pourrat, J., Seligman, R., Fridman, W. H., Loirat, C. & Atkinson, J. P. (2006) Genetic and functional analyses of membrane cofactor protein (CD46) mutations in atypical hemolytic uremic syndrome. *J Am Soc Nephrol* **17**, 2017–2025.

George, J. N. (2006) Clinical practice. Thrombotic thrombocytopenic purpura. *N Engl J Med* **354**, 1927–1935.

Goicoechea De Jorge, E., Harris, C. L., Esparza-Gordillo, J., Carreras, L., Arranz, E. A., Garrido, C. A., Lopez-Trascasa, M., Sanchez-Corral, P., Morgan, B. P. & Rodriguez De Cordoba, S. (2007) Gain-of-function mutations in complement factor B are associated with atypical hemolytic uremic syndrome. *Proc Natl Acad Sci U S A* **104**, 240–245.

Heinen, S., Sanchez-Corral, P., Jackson, M. S., Strain, L., Goodship, J. A., Kemp, E. J., Skerka, C., Jokiranta, T. S., Meyers, K., Wagner, E., Robitaille, P., Esparza-Gordillo, J., Rodriguez De Cordoba, S., Zipfel, P. F. & Goodship, T. H. (2006) De novo gene conversion in the RCA gene cluster (1q32) causes mutations in complement factor H associated with atypical hemolytic uremic syndrome. *Hum Mutat* **27**, 292–293.

Kaplan, B. S., Meyers, K. E. & Schulman, S. L. (1998) The pathogenesis and treatment of hemolytic uremic syndrome. *J Am Soc Nephrol* **9**, 1126–1133.

Kavanagh, D., Kemp, E. J., Mayland, E., Winney, R. J., Duffield, J. S., Warwick, G., Richards, A., Ward, R., Goodship, J. A. & Goodship, T. H. (2005) Mutations in complement factor I predispose to development of atypical hemolytic uremic syndrome. *J Am Soc Nephrol* **16**, 2150–2155.

Kavanagh, D., Richards, A. & Atkinson, J. (2008) Complement regulatory genes and hemolytic uremic syndromes. *Annu Rev Med* **59**, 293–309.

Lahlou, A., Lang, P., Charpentier, B., Barrou, B., Glotz, D., Baron, C., Hiesse, C., Kreis, H., Legendre, C., Bedrossian, J., Mougenot, B., Sraer, J. D. & Rondeau, E. (2000) Hemolytic uremic syndrome. Recurrence after renal transplantation. Groupe Cooperatif de l'Ile-de-France (GCIF). *Medicine (Baltimore)* **79**, 90–102.

Miller, R. B., Burke, B. A., Schmidt, W. J., Gillingham, K. J., Matas, A. J., Mauer, M. & Kashtan, C. E. (1997) Recurrence of haemolytic-uraemic syndrome in renal transplants: a single-centre report. *Nephrol Dial Transplant* **12**, 1425–1430.

Neumann, H. P., Salzmann, M., Bohnert-Iwan, B., Mannuelian, T., Skerka, C., Lenk, D., Bender, B. U., Cybulla, M., Riegler, P., Konigsrainer, A., Neyer, U., Bock, A., Widmer, U., Male, D. A., Franke, G. & Zipfel, P. F. (2003) Haemolytic uraemic syndrome and mutations of the factor H gene: a registry-based study of German speaking countries. *J Med Genet* **40**, 676–681.

Ng, P. C. & Henikoff, S. (2006) Predicting the effects of amino acid substitutions on protein function. *Annu Rev Genomics Hum Genet* **7**, 61–80.

Noris, M., Brioschi, S., Caprioli, J., Todeschini, M., Bresin, E., Porrati, F., Gamba, S. & Remuzzi G. (2003) Familial haemolytic uraemic syndrome and an MCP mutation. *Lancet* **362**, 1542–1547.

Rey-Campos, J., Rubinstein, P. & Rodriguez De Cordoba, S. (1988) A physical map of the human regulator of complement activation gene cluster linking the complement genes CR1, CR2, DAF, and C4BP. *J Exp Med* **167**, 664–669.

Richards, A., Buddles, M. R., Donne, R. L., Kaplan, B. S., Kirk, E., Venning, M. C., Tielemans, C. L., Goodship, J. A. & Goodship, T. H. (2001) Factor H mutations in hemolytic uremic syndrome cluster in exons 18–20, a domain important for host cell recognition. *Am J Hum Genet* **68**, 485–490.

Richards, A., Kemp, E. J., Liszewski, M. K., Goodship, J. A., Lampe, A. K., Decorte, R., Muslumanoglu, M. H., Kavucku, S., Filler, G., Pirson, Y., Wen, L. S., Atkinson, J. P. & Goodship, T. H. (2003) Mutations in human complement regulator, membrane cofactor protein (CD46), predispose to development of familial hemolytic uremic syndrome. *Proc Natl Acad Sci U S A* **100**, 12966–12971.

Rock, G. A., Shumak, K. H., Buskard, N. A., Blanchette, V. S., Kelton, J. G., Nair, R. C. & Spasoff, R. A. (1991) Comparison of plasma exchange with plasma infusion in the treatment of thrombotic thrombocytopenic purpura. Canadian Apheresis Study Group. *N Engl J Med* **325**, 393–397.

Sanchez-Corral, P., Perez-Caballero, D., Huarte, O., Simckes, A. M., Goicoechea, E., Lopez-Trascasa, M. & De Cordoba, S. R. (2002) Structural and functional characterization of factor H mutations associated with atypical hemolytic uremic syndrome. *Am J Hum Genet* **71**, 1285–1295.

Saunders, R. E., Goodship, T. H., Zipfel, P. F. & Perkins, S. J. (2006) An interactive web database of factor H-associated hemolytic uremic syndrome mutations: insights into the structural consequences of disease-associated mutations. *Hum Mutat* **27**, 21–30.

Vyse, T. J., Morley, B. J., Bartok, I., Theodoridis, E. L., Davies, K. A., Webster, A. D. & Walport, M. J. (1996) The molecular basis of hereditary complement factor I deficiency. *J Clin Invest* **97**, 925–933.

Warwicker, P., Goodship, T. H., Donne, R. L., Pirson, Y., Nicholls, A., Ward, R. M., Turnpenny, P. & Goodship, J. A. (1998) Genetic studies into inherited and sporadic hemolytic uremic syndrome. *Kidney Int* **53**, 836–844.

Xiao, W. & Oefner, P. J. (2001) Denaturing high-performance liquid chromatography: A review. *Hum Mutat* **17**, 439–474.

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